15 "Metallomics" — a Multidisciplinary Metal-Assisted Functional Biogeochemistry: Scope and Limitations

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15.1 INTRODUCTION

"Metallomics," a metal-assisted functional biogeochemistry, is a new scientific field first proposed by Haraguchi [1] to integrate the research fields related to metal biomolecules and its metals. Metallomics is the scientific field of symbiosis with genomics and proteomics because syntheses and metabolic functions of genes (DNA and RNA) and proteins cannot function without the

TABLE 15.1
Typical Metalloenzymes (and Metalloproteins) and Their Biological Functions

Metalloenzyme (MWa/kDa)	Number of atoms	Biological function
Transferrin (66–68)	2Fe	Transportation of iron
Ferritin (473)	1Fe	Storage of iron
Catalase (225)	4Fe	Decomposition of H_2O_2
Nitrogenase (200–220)	24Fe, 2Mo	Nitrogen fixation
Chitochrome P-450 (50)	Fe	Metabolisms of steroids and drugs
Carbonic anhydrase(30)	1Zn	Catalyst of H_2CO_3 equilibrium
Carboxypeptidase (34)	1Zn	Hydrolysis of peptide bonds at carboxy terminal
Alcohol dehydogenase (150)	4Zn	Dehydration of alcohol
Alkaline phosphatase (89)	3.5Zn	Hydrolysis of phosphate esters
DNA polymerase (109)	2Zn	DNA synthesis
RNA polymerase (370)	2Zn	RNA synthesis
Plastocyaneine (134)	1Cu	Electron transfer
Gluthathion peroxidase (76–92)	1Se	Decomposition of H_2O_2 and organic superoxides
Urease (480)	10Ni	Transformation of urease to ammonia

Source: Haraguchi, H., *J. Anal. At. Spectrum*, 19, 5, 2004. With permission.

coordination of various metal ions and metalloenzymes. In metallomics, metalloproteins, metalloenzymes, and other metal-containing biomolecules are defined as "metallomes" in a similar manner to genomes in genomics as well as proteomes in proteomics. Because the identification of metallomes and the elucidation of their biological or physiological functions in biological systems is the main research target of metallomics, chemical speciation for specific identification of bioactive metallomes is the crux of establishing metallomics as an integrated biometal science.

Hazardous or toxic elements such as Hg, Cd, Pb, Cr(VI), As, Sn, and Se have caused serious environmental pollution or toxicological problems; thus, such elements in the biological, environmental, and geochemical samples have been extensively determined for environmental management and/or protection from environmental hazards, Most of trace metals in biological fluids and organs bind with various proteins called "metalloproteins." Metalloproteins are called "metalloenzymes" when they work as the biological catalysts to regulate biological reactions and physiological functions in biological cells and organs.

Some typical metalloenzymes and metalloproteins are summarized in Table 15.1. Metalloenzymes contain the specific number of metal ions at the active sites in specific proteins and they work as biocatalysts for specific enzymatic reactions, including gene (DNA, RNA) synthesis; metabolism; antioxidation; and so forth. In addition, the bioavailability and toxicity of the elements also depend on their chemical forms. Thus, species analysis of metal-binding molecules in biological samples is an important subject in various scientific fields such as biochemistry; biology; medicine; pharmacy; nutrition; agriculture; environmental science; etc. Accordingly, in recent years, chemical speciation or elemental speciation has been extensively developed to elucidate the biological essentiality and toxicity of the elements on a molecular basis [1,2,10–17]. Highly sensitive analytical methods such as AAS (atomic absorption spectrometry); ICP-AES (inductively coupled plasma atomic emission spectrometry); ICP-MS (inductively coupled plasma mass spectrometry); XRF (x-ray fluorescence spectrometry); and NAA (neutron activation analysis) enable one to determine almost all elements in the major to ultratrace concentration ranges.

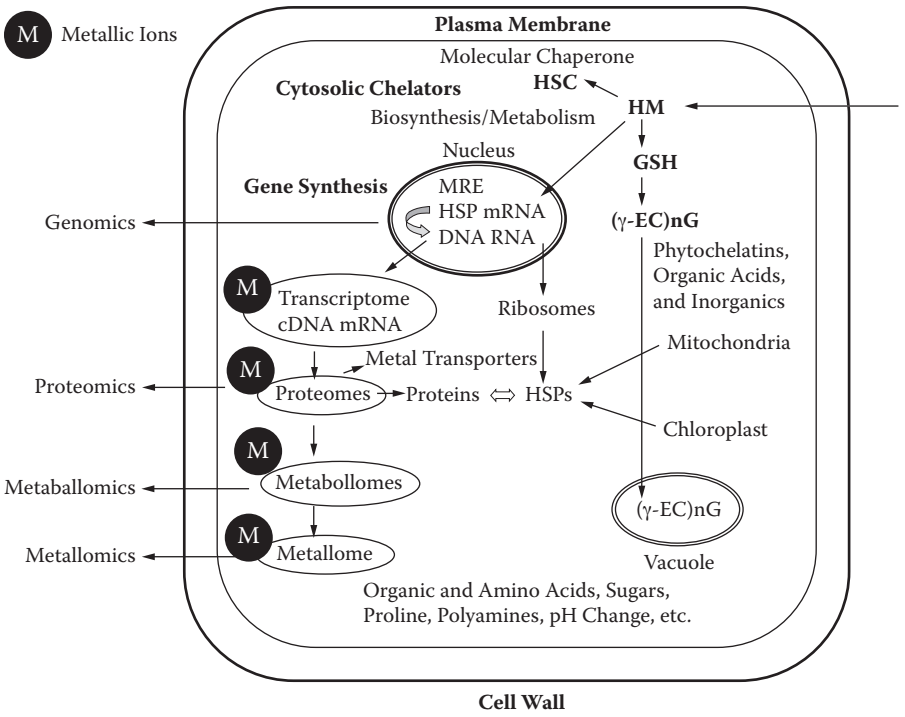

FIGURE 15.1 Basic principles of biological chemistry and proteomics for metabolic engineering of appropriate plants to control and transform hazardous pollutants. HM = heavy metal; M = metal ion; MRE = metal regulatory element; HSP = heat shock protein; HSC = heat shock cognate; ((-EC)nG – Glu – Cys- Gly aminoacids).

15.2 METALLOMICS AND METALLOMES

A schematic sketch of metallomics is proposed in Figure 15.1. On the left-hand side, academic technical terms such as genomics, proteomics, and metabollomics are shown along with metallomics to indicate their research areas in the biological system. Genomics deals with the scientific works on the genetic information of DNAs and RNAs encoded as the sequences of nucleic bases. DNA and RNA play an essential role in protein synthesis. Proteins are distributed inside and outside the cell, and they work as enzymes for synthesis and metabolism of various biomolecules of the cell. It is seen that a large number of proteins play essential roles in syntheses and metabolisms of many biological molecules to regulate and maintain the life system; protein science has been receiving great attention as postgenome science linked with genomics.

Many biological substances as well as metal ions are transported as raw materials inside the cell through the membrane. In general, material conversion is actively occurring inside the cell and also often in the cell membrane, and such material conversion and transportation in evolving specific transporters is termed "metabolism." Biological substances, which are usually small molecules such as amino acids, organic acids, and metal ions produced in metabolism, have recently been called "metabollomes" or "metabolites." Bioscience concerned with metallic elements and their applications has been studied independently in many scientific fields such as biochemistry; bioinorganic chemistry; nutritional science; pharmacy; medicine; toxicology; agriculture; and environmental science.

All such scientific fields have a deep interrelationship, with the common factor of metals, from the viewpoint of biological science. Therefore, it is desirable to promote it as an interdisciplinary field. Thus, Haraguchi [1] proposed the nomenclature of "metallomics" for biometal science. In

the study of metallomics, elucidation of the physiological roles and functions of biomolecules binding with metallic ions in the biological systems should be the most important research target.

In recent years, genomics and proteomics have received great attention to appreciate various biological systems from the viewpoints of gene and protein sciences. Genomics and proteomics are indeed fundamentally important scientific fields because genes (DNAs and RNAs) contain the genetic information codes to synthesize various proteins. Genes and proteins cannot be synthesized without the assistance of metalloenzymes containing zinc and other metals. In this sense, metallomics may stand in the same position in scientific significance as genomics and proteomics. Thus, in metallomics, biological molecules bound with biometals are properly defined as "metallomes," corresponding to genomes and proteomes in genomics and proteomics, respectively. However, metallic ions such as alkali and alkaline earth metal ions, which exist mostly as free ions in biological fluids, should also be included in metallomes because they play many important roles in the occurrence of the physiological functions in the biological systems.

15.3 GLUTATHIONE AND ORGANIC ACIDS METABOLISM

Glutathione and organic acids metabolism plays a key role in metal tolerance in plants [2–5]. Glutathione is ubiquitous component cells from bacteria to plants and animals. In plants, it is the major low molecular mass thiol compound (28). Glutathione occurs in plants mainly as reduced GSH (95 %). Its synthesis is mediated by the enzymes glutamylcysteine synthetase (EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3). Glutathione metabolism is also connected with cysteine and sulphur metabolism in plants. Cysteine concentration limits glutathione biosynthesis. Low molecular thiol peptide phytochelatins (PCs), often called class III metallothioneins, are synthesized in plants from glutathione induced by heavy metal ions [6].

These peptides are synthesized from glutathione by means of α-glutamylcysteine transferase enzyme (EC 2.3.2.15), which is also called phytochelatin synthase (PCS), catalyzing transfer reaction of (α-Glu-Cys) group from a glutathione donor molecule to glutathione, an acceptor molecule. PCS is a cytosolic, constitutive enzyme and is activated by metal ions, namely, Cd^{2+}, Pb^{2+}, Ag^{1+}, Bi^{3+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, and Au^{2+}. PCs thus synthesized chelate heavy metals and form complexes that are transported through cytosol in an ATP-dependent manner through tonoplast into vacuole. Thus, the toxic metals are swept away from cytosol. Some high molecular weight complexes (HMW) with S-2 can also be formed from these LMW complexes in vacuole [7].

Transgenic plants with modified genes of PCS and genes of glutathione synthesis enzymes, α-GCS and GS, and enzymes connected with sulphur metabolism, e.g., serineacetyltransferase, need special attention in order to achieve success in phytoremediation of metals in the environment. Plants under heavy metal stress produce free radicals and reactive oxygen species and must withstand the oxidative stress before acquiring tolerance to toxic metals. Glutathione is then used for the synthesis of PCs as well as for dithiol (GSSG) production. The ascorbate–glutathione pathway is involved in plant defense against oxidative stress. Organic acids play a major role in metal tolerance [8].

Organic acids play a role in metal chelation by forming complexes with metals, a process of metal detoxification. Chelation of metals with exuded organic acids in the rhizosphere and rhizospheric processes indeed form an important aspect of investigation for remediation. These metabolic pathways underscore the physiological, biochemical, and molecular bases for heavy metal tolerance [6].

15.4 METAL TRANSPORTERS AND INTERACTIONS IN MEMBRANES AT MOLECULAR LEVELS

Plants and humans require adequate amounts of micronutrients like iron and zinc, but accumulation of an excess or uptake of nonessential metals like cadmium or lead can be extremely harmful.

Proteins of the CDF (cation diffusion facilitator) family are involved in the homeostasis of Cd^{2+}, Co^{2+}, Fe^{2+}, and Zn^{2+} in microbes, animals, and plants [9]. Therefore, elucidation of the role of CDF proteins in *Arabidopsis thaliana* would be advantageous to the success of phytoremediation. Complementary DNAs are to be functionally expressed in appropriate mutants of *Saccharomyces cerevisiae* to test their function.

In a reverse genetics approach, several representative *Arabidopsis* CDFs will be used in RNA interference technology [10,11]. Regulation and localization of these CDFs need to be investigated by expressing promoter:GUS fusions and epitope-tagged fusion proteins in *A. thaliana*, and by development and use of specific antibodies. Very little information is available about protein–protein interactions of membrane. Such interactions might be vital for CDF function because their substrate metal cations are thought to be bound to metallochaperone proteins in the cytoplasm.

15.5 SPECIES-SELECTIVE ANALYSIS FOR METALS AND METALLOIDS IN PLANTS

The success of an analyst searching for a given organometallic moiety in a plant matrix depends on two factors. First, he or she must be sure to determine this and not another species (analytical selectivity). Second, the detection limit (sensitivity of the detector and noise level) of the instrumental setup should match the analyte's level in the sample.

Intrinsically, species-selective techniques, such as Mössbauer spectroscopy; x-ray photoelectron spectroscopy (XPS); electron spin resonance spectroscopy (ESR); or mass or tandem mass spectrometry (MS or MS/MS) usually fail at trace levels in the presence of a real-sample matrix. Al (70.4 MHz) NMR spectra were obtained on various intact samples of Al-accumulating plant tissues. None of the materials examined (*Hydrangea sepals* and leaves of three Theaceae species) contained detectable amounts of $Al(H_2O)_6^{3+}$, although the Al was present as hexacoordinated complexes and, in most instances, in at least two forms.

Selectivity in terms of species is typically achieved by on-line combination of a high-performance separation technique (chromatography or electrophoresis) with the parallel element-specific and molecule-specific detection. Nonspecific detectors [UV, flame ionization detector (FID)] suffer from a large background noise and poor sensitivity. Analysis by a coupled technique is often preceded by a more or less complex wet chemical sample preparation. The latter is mandatory for the sample to meet the conditions (imposed by the separation technique) in terms of form to be presented to the instrumental system. The tendency is to integrate this preliminary step into the whole experimental setup.

The coupled techniques available for elemental speciation analysis have been reviewed elsewhere (Table 15.2) [12]. The choice of the separation technique is determined by the physicochemical properties of the analyte (volatility, charge, polarity); that of the detection technique is determined by the analyte's level in the sample. This is the sample matrix (air, water, sediment, biomaterial) that dictates, on its turn, the choice of the sample preparation procedure. Separation techniques for speciation analysis have been comprehensively reviewed [12]. For volatile species or those convertible readily to volatile ones by means of derivation, gas chromatography (GC) is the method of choice. Species that do not fulfill the preceding requirement are separated by ion exchange or reversed-phase liquid chromatography. In particular, proteins and other biopolymers are separated by size-exclusion (gel-permeation) chromatography.

The physicochemical similarity of many proteins stimulates the use of electrophoretic techniques for efficient separations. In terms of detectors, plasma spectrometric techniques are favored over atomic absorption spectrometry (AAS) because of their much higher sensitivity. Microwave-induced plasma (MIP) atomic emission spectrometry is the choice for GC and a more energetic inductively coupled plasma (ICP) mass spectrometry is the choice for LC and capillary zone electrophoresis (CZE).

Element-specific detectors do not allow for the identification of the species eluted. This drawback gains in importance as the wider availability of more efficient separation techniques and more sensitive detectors makes the number of unidentified species grow. Therefore, the prerequisite for progress in speciation analysis is a wider application of sensitive on-line techniques for compound identification, i.e. mass or tandem mass spectrometry with soft (e.g., electrospray (ESI) or matrix-assisted laser desorption ionization (MALDI).

Thus far, the hyphenated techniques such as LC-ICP-MS, GC-ICP-MS, and LC-ICP-AES have been developed as the analytical methods for chemical speciation of trace metals in the biological samples. Such hyphenated methods are actually the most powerful techniques for chemical speciation. However, they are useful only for the identification of known and stable compounds such as methylmercury, methylated arsenics, butyltin compounds, and so on. From now on, however, it is obvious that the identification of biomolecules such as metalloproteins and metalloenzymes, as well as metal-binding nucleic acids and metabolites, will become more important in exploring biometal science in relation to their biological functions and metabolism. Then, the methods for the direct identification of biomolecules, such as ES-MS (electrospray mass spectrometry) and MALDITOFMS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry), should be employed for study of chemical speciation.

Perhaps a system of LC doubly combined with ES-MS (or MALDI-TOFMS) and ICP-MS — i.e., LC-ES-MS-ICP-MS, which is a kind of tandem mass spectrometry and allows detection of organic molecules and trace metals simultaneously — may be the more ideal instrumentation for direct speciation of unknown organometallic compounds and metalloproteins to promote development and establishment of metallomics. It has been noted that the detection sensitivities for organic molecules obtained by organic mass spectrometry presently seem to be inferior to those for trace metals by ICP-MS. Therefore, the sensitivity matching in the hyphenated system, for example, using ES-MS and ICPMS, should be explored by improving the ionization efficiencies of bioorganic molecules in organic mass spectrometry to develop such a simultaneous detection system.

15.6 METABOLLOMICS

15.6.1 Glutathione Metabolism and Phytochelatin Synthesis

Phytochelatin synthase (PCS) genes overexpressed in a PCS-positive background or transplanted into plants that lack an endogenous PCS homolog would serve as reliable biotechnological and molecular tools for heavy metal remediation in the environment. However, an integrated investigation of phytochelatin biosynthesis is warranted to understand the potential and limitations of a PCS-based metal detoxification as phytoremediation strategy [13]. Phytochelatins are considered to be the activated sulfate acceptors in the formation of a thiosulfate intermediate leading to sulfite formation upon reduction by thiosulfonate reductase [14]. This hypothesis, however, has been challenged by

- The fact that no plant thiosulfonate reductase has been identified thus far
- The recent demonstration that the main sulfite-forming pathway in plants relies on an enzyme adenosine 5′-phosphosulfate reductase (APS) that directly reduces activated sulfate using an intramolecular glutaredoxin domain [15]

15.7 CHEMICAL TRANSFORMATION

Selenate, the oxoanion of the element selenium, is taken up and metabolized by higher plants because of its chemical similarity to sulfate. Thus, growth on soils contaminated with selenate results in the formation of excess amounts of selenocysteine and selenomethionine. These are

TABLE 15.2
Research Subjects in Metallomics and Analytical Techniques Required in Metallomics Researches

Research subject	Analytical technique
Distributions of the elements in the biological fluids, cell, organs, etc.	Ultratrace analysis, all-elements analysis, one atom detection, one molecule detection
Chemical speciation of the elements in the biological samples and systems	Hyphenated methods (LC-ICP-MS, GC-ICP-MS, MALDI-MS, ES-MS)
Structural analysis of metallomes (metal-binding molecules)	X-ray diffraction analysis, EXAFS
Elucidation of reaction mechanisms of metallomes using model complexes (bioinorganic chemistry)	NMR, XPS, laser-Raman spectroscopy, DNA sequencer, amino acids sequencer, time-resolution and spatial-resolution fluorescence detection
Identification of metalloproteins and metalloenzymes	LC-ES-MS, LC-MALDI-MS, LC-ICP-MS
Metabolisms of biological molecules and metals (metabollomes, metabolites)	LC, GC, LC-MS, GC-MS, ES-MS, API-MS[a], biosensors
Medical diagnosis of health and disease related to trace metals on a multielement basis	ICP-AES, ICP-MS, graphite-furnace AAS, autoanalyzer, spectrophotometry
Design of inorganic drugs for chemotherapy	LC-MS, LC-ICP-MS, stable isotope tracers
Chemical evolution of living systems and organisms on the Earth	Isotope ratio measurement (chronological techniques, DNA sequencer
Other metal-assisted function biosciences in medicine, environmental science, food science, agriculture, toxicology, biogeochemistry, etc.	*In-situ* analysis, immunoassay, bioassay, food analysis, clinical analysis

[a] Atmospheric pressure chemical ionization mass spectrometry.

Source: Haraguchi, H., *J. Anal. At. Spectrum*, 19, 5, 2004. With permission.

incorporated into proteins of sensitive plants instead of cysteine and methionine and render the affected proteins nonfunctional.

In an approach to increase selenium assimilation by plants, the plastidic *A. thaliana* APS1 cDNA-encoding ATP sulfurylase was expressed in *B. juncea* under the control of a 35S promoter [16]. Transgenic plants exhibited a slightly increased tolerance to selenate when compared with wild-type controls and accumulated approximately twofold higher concentrations of selenium in their shoots. Enhanced sulfur assimilation in these transgenic plants resulted in an increase in glutathione concentrations by approximately 100 and 30% in shoots and roots, respectively; this suggested that ATP sulfurylase might also be an interesting target for the phytoremediation of other metals, especially cadmium [17].

Chemical transformation of a trace element into a less toxic, volatile compound is a very effective strategy for detoxification because the potentially harmful element is removed from the tissues. In mercury-contaminated soils and sediments, microbial activity results in the conversion of toxic Hg(II) into organomercurials — for example, the highly toxic methylmercury (CH_3 Hg^+). Mercury-resistant bacteria able to transform organomercurials and Hg(II) into significantly less toxic elemental mercury have been isolated. Methylmercury is converted to the less toxic Hg(II) by organomercurial lyase encoded by the gene *MerB*. A second enzyme, encoded by *MerA*, catalyzes the reduction of Hg(II) to elemental mercury, using NADPH as the electron donor. Under ambient conditions, elemental mercury enters the global biogeochemical cycle upon volatilization [18].

Mercury volatilization in plants has been established [16,18,19]. The nucleotide sequence of a bacterial *MerA* gene had to be modified to allow for high-level expression in plants. *Arabidopsis thaliana* expressing *Mer A* under the control of a constitutive cauliflower mosaic virus 35S promoter germinated and developed on agarose media containing 50 and 100 μM $HgCl_2$, concentrations that

completely inhibited germination of wild-type seeds. The *MerA* plants showed a significantly higher tolerance to Hg^{2+} and volatalized Hg; their tolerance to methylmercury was unchanged. They were also more tolerant to Au^{3+}.

The *MerB* plants were significantly more tolerant to methylmercury and other organomercurials. They effectively converted highly toxic methylmercury to Hg^{2+}, which is 100 times less toxic to plants. To study the effects of both, the *MerA* and *MerB* plants were crossed and F1 generation was selfed. The F2–*MerA*, *MerB* double transgenics showed highest tolerance to organic mercury (10 micro molar = (uM)). *MerA* and *MerB* plants were shown to volatilize elemental mercury when supplied with organic mercury. Submicromolar concentrations of highly toxic organomercurials abolish germination of wild-type and *MerA*-expressing *A. thaliana*.

The combined expression of *MerA* and *MerB* in a high-biomass plant could be a promising step towards the generation of an improved mercury phytoremediator plant. Modified *MerA* genes were then introduced into higher biomass plants. Tobacco transformants expressing a modified *MerA* gene were able to develop and flower on soils containing up to 500 ppm Hg(II), but mercury removal from soil substrates has yet to be determined [18].

15.8 SULPHUR METABOLISM

Sulfur metabolism and HM detoxification are closely related processes [7] (see Chapter 16) (Figure 15.2). In addition, a possible homeostatic role of PCs towards essential HMs is clearly indicated by the fact that (1) low, but detectable, levels of PCs are present even in the absence of HM exposure; and (2) PC levels increase concomitantly with Cu and Zn depletion upon transfer of cell suspension cultures to a minimal micronutrient medium. In fact, it has been hypothesized that PCs are not simply an HM-detoxification system *sensu stricto*, especially in the presence of low concentrations of (essential) metals. Instead, under these conditions, PCs may primarily act as key components of metal homeostasis. The constitutive expression of PCS [20] might also be considered as an indication of a more general role of PCs not exclusively related to HM detoxification.

Further supporting this view is the strong protective effect of PCs against Cd-mediated inactivation of metal-sensitive enzymes and the ability of Zn– and Cu–PC complexes, mainly of the PC_2/PC_3 type, to reactivate metal-depleted or metal-poisoned metalloenzymes (although not more efficiently than the corresponding free salts). In plants, it is thus possible that PCs and MT-like proteins cooperatively act in the homeostasis of essential HMs. PCs have also been proposed as activated sulfate acceptors in the formation of a thiosulfate intermediate leading to sulfite formation upon reduction by thiosulfonate reductase. This hypothesis, however, has been challenged by the fact that no plant thiosulfonate reductase has been identified thus far and by the recent demonstration that the main sulfite-forming pathway in plants relies on an enzyme (adenosine 5′-phosphosulfate reductase) that directly reduces activated sulfate (APS) using an intramolecular glutaredoxin domain [15].

Although the metal detoxification and homeostatic roles of PCs are not mutually exclusive and may coexist at the whole plant level, the fact that *Arabidopsis* PC-deficient mutants (*cad1*) grow well in the presence of Cu and Zn micronutrient concentrations [21] suggests that the latter role, if real, is dispensable or easily replaceable by other metal-binding components, such as MTs. On the other hand, the idea of an exclusive metal detoxification function of PCs is somewhat weakened by the lack of correlation between the PC content and the HM sensitivity of metal-tolerant and nontolerant ecotypes of *Silene vulgaris* and *Datura innoxia* and by similar findings recently reported for the HM hyperaccumulator *Thlaspi caerulescens* and the closely related, nonaccumulator species *Thlaspi arvense* [22–24]. Neither PCS activity nor PC turnover upon transfer to a Cd-less medium differed between wild-type and HM-tolerant *Silene vulgaris* [15]. In addition, although PC_2 was the most abundant PC peptide in metal-tolerant plants, the more effective metal chelator, PC_3, prevailed in the nontolerant ecotype [26].

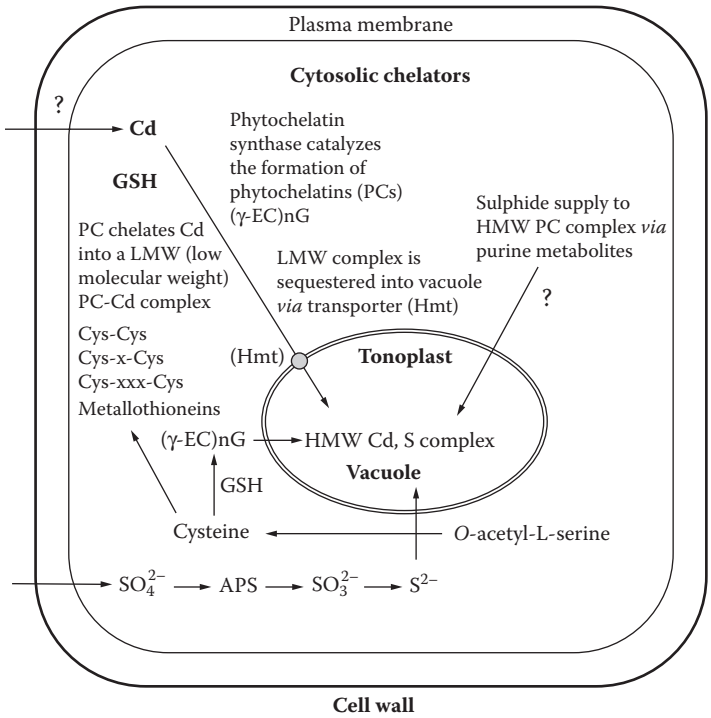

FIGURE 15.2 Sulfur metabolism and HM detoxification mechanisms — a comprehensive view.

Further to this point, a stronger correlation between Cu tolerance and the accumulation of MT mRNAs (r values ranging from 0.89 to 0.998) than with the total amount of intracellular nonprotein thiols, including PCs (r = 0.77), has been reported in *Arabidopsis* [27]. Therefore, it cannot be excluded that other systems, autonomously or in combination with PCs, may regulate HM homeostasis in the plant cell. For instance, Cu-MTs and Cu chaperones (termed Atx1, Lys7, and Cox 17) seem to be involved in Cu ion traffic in yeast cells [28] and analogous mechanisms might operate in higher plants [29].

Multiple connections exist between sulfur metabolism and heavy metal detoxification and homeostasis in plants. Metal ions are complexed in the cytosol with GSH and the derived PCs are transported into vacuole. The following functions have been implicated in Cd complexation and transport [30 and the references therein]:

- The ATP-binding cassette-type transport activity at the tonoplast
- The Cd^{2+}/H^+ antiport
- The vacuolar-type ATPase generating a proton gradient

Another notable cellular response depicted is that some metals interact with genes that have metal-regulating elements (MRE) at the promoter region, as found for MT genes of animals. For example, in the soybean, similar sequences have been found in the upstream region of heat shock gene coded for 17.5 kDa HSP (heat shock protein)[31]. Some heat shock genes and small HSPs have been induced by Cd ions in soybean [31,32]. These HSCs (heat shock cognates) thus may function as molecular chaperones [33,34].

Cui et al. [35] reported that the elemental sulphur and EDTA amendments increased the extractable fractions of soil Pb and Zn. EDTA was more effective than S. Shoot uptake of Pb and

Zn by Indian mustard and wheat was increased with S and EDTA amendments. Indian mustard shoots took up more Pb and Zn than winter wheat with or without S and EDTA amendment.

15.9 OVEREXPRESSION OF LCT1 (LOW-AFFINITY CATION TRANSPORTER) IN TOBACCO ENHANCED: THE PROTECTIVE ACTION OF CALCIUM AGAINST CADMIUM TOXICITY

Metal–metal interactions have been reported to have ameliorating functions [36–40]. For example, calcium involvement in zinc uptake and detoxification was studied in Zn^{2+}-tolerant and nontolerant populations of *Silene maritima.* Increasing calcium concentrations reduced Zn toxicity and led to a higher level of zinc accumulation by the roots of the tolerant plants; however, they decreased transport to the shoots of both types [41]. A higher calcium concentration in a medium was also reported to abolish the toxic effects of Cd^{2+} [42,43] and Pb^{2+} [44] on the activity of photosystem II. In addition, high Ca status and a high level of tolerance to Ca deficit accompanied enhanced Zn, Pb, Cu, and Al tolerance [45–47].

The regulation of heavy metal uptake and internal transport constitutes part of the basis of plant resistance to their toxicity. The mechanism accounting for the transport of heavy metals across membranes in plants and its regulation is far from understood, however. The general view is that nonessential metals usually cross plasmalemma and internal membranes through cation transporters with a broad substrate specificity or that they use pathways reserved for other ions [48,49]. For example, cadmium and lead were shown to be transported through pathways for calcium ions.

In plants, it has been suggested that putative tonoplast Ca^{2+}/H^{+} antiporters encoded by CAX1 and CAX2 (calcium exchange protein) from *Arabidopsis* are involved in the transport of cadmium from the cytoplasm to the vacuole [50]. In turn, LCT1 cloned from wheat roots (however expressed in roots and leaves), a nonselective transmembrane transporter for Na^{+}, K^{+} [51,52], and Ca^{2+}, also appeared to mediate Cd^{2+} transport to the cell [53]. Toxic metal ions such as Cd^{2+} or Pb^{2+} are known as very effective substituents of Ca^{2+}, e.g., in calmodulin, consequently interfering seriously with the role of this cation in a number of metabolic processes [54–56]. In this context, it seems possible that the regulation of heavy metal uptake/transport in a plant by the presence of calcium in various concentrations in the medium could in part contribute to an ameliorative effect of calcium on heavy metal toxicity.

To gain insight into the molecular mechanism of Ca^{2+}-dependent Cd^{2+} tolerance, tobacco was transformed with wheat cDNA LCT1, the first cloned plant influx system mediating the uptake of Ca^{2+} and Cd^{2+} ions into a cell [53]. Transformants were tested for the possible involvement of LCT1 in diminishing Cd toxicity with the enhancement of Ca^{2+} concentration in the medium. Antosiewicz and Hennig [57] also demonstrated that LCT1 is involved in calcium acquisition and in the alleviation of toxic effects of Cd^{2+} by enhanced external Ca^{2+} concentration.

Antosiewicz and Hennig [57] were the first to demonstrate the involvement of LCT1 in calcium acquisition and in the regulation of amelioration of Cd toxicity by calcium. Wheat cDNA LCT1 (low-affinity cation transporter gene), a nonspecific transporter for Ca^{2+}, Cd^{2+}, Na^{+}, and K^{+}, was overexpressed in tobacco. Transformants were tested for their sensitivity to a range of Ca^{2+} concentrations [0.01 to 10 m*M* Ca(NO3)2] with or without the presence of 0.05 m*M* Cd(NO3)2. LCT1-transformed plants expressed a phenotype distinct from controls only under conditions of low calcium (0.01 to 1 m*M* Ca^{2+}). They grew significantly better and had slightly higher shoot calcium concentration.

Transformants subjected to 0.05 m*M* Cd(NO3)2 in the presence of 1 m*M* Ca^{2+} displayed a substantially higher level of tolerance to cadmium and accumulated less Cd in roots. LCT1 increased calcium and decreased cadmium accumulation in transformed plants. LCT1, the putative plasmalemma nonspecific transporter for Ca^{2+}, Cd^{2+}, Na^{+}, and K^{+} [51–53], was used for tobacco transformation in order to check whether the toxicity of cadmium administered in a range of Ca^{2+} concen-

trations to control and LCT1-transformed plants would be different. LCT1 improved plant performance at low external calcium; LCT1 expression in tobacco improved the growth of plants only within a limited range of calcium concentrations. LCT1 contributed to Ca^{2+}-dependent Cd^{2+} tolerance.

Numerous authors have described the phenomenon of calcium mitigating heavy metal toxicity [36,41–44]. The reported amelioration constitutes a part of the whole-plant defense system that includes uptake/transport and detoxification/sequestration. Based on the known broad spectrum of the role of Ca^{2+} in the regulation of metabolic processes [58], calcium might be an important factor in any of these components. It is not known whether the observed reduced toxicity might result from less cadmium uptake or from more efficient detoxification. For example, Choi et al. [59] demonstrated the contribution of calcium to the protection mechanism by immobilization of the metal as coprecipitates with calcium and phosphorous. Reduction of cadmium uptake and accumulation by calcium were also reported for several plant species [60–62] as was the opposite effect: inhibition of the accumulation of calcium by cadmium, leading to calcium deficiency [63].

15.10 OVEREXPRESSION OF ALFALFA ALDOSE/ALDEHYDE REDUCTASE CONFERS TOLERANCE TO CADMIUM STRESS

Wild-type (SR1) tobacco line and transgenic lines ALR1/5 and ALR1/9 overexpressing aldose/aldehyde reductase were less susceptible to cadmium-induced stress. Based on these observations, Hegedüs et al. [64] suggested that alfalfa aldose/aldehyde reductase overexpression may generally induce higher stress tolerance. These coworkers transformed tobacco (*Nicotiana tabacum* cv. Petit Havanna line SR1) by alfalfa aldose/aldehyde reductase cDNA attached to the viral constitutive promoter CaMv35S. The WT (SR1), a nonexpressing transgenic line (ALR1/7), and two transgenic lines (ALR1/5 and ALR1/9) previously shown by Western hybridization to overexpress the alfalfa ALR protein [21] were used in these experiments.

The ectopic synthesis of a novel alfalfa NADPH-dependent aldose/aldehyde reductase enzyme in transgenic tobacco plants provided substantial tolerance against oxidative stress, such as drought, paraquate, and UV-B [21,22]. Cd treatment caused a significant decrease in the chlorophyll content in all the genotypes tested. However, this decrease was less pronounced in the lines overproducing aldose/aldehyde reductase (72 and 74% for lines ALR1/9 and ALR1/5, respectively) than in the SR1 wild-type plants (52%). Similar tendencies were revealed for changes in the total carotenoid content of tobacco leaves.

The novel molecular methodologies, i.e., genomics and proteomics, have not been brought together in the past to characterize molecular mechanisms related to plant metal accumulation. Because the *Arabidopsis* genome has been completely sequenced, the full benefit of those data is available for identification of genes and proteins found in plants that hyperaccumulate metals. Various ecotypes of plants that hyperaccumulate metals are being currently investigated in various laboratories to identify metal responsive proteins. Proteomics provides a powerful additional tool for the identification of proteins induced or repressed under metal stress and also for comparison of various ecotypes. *Thlaspi* is the ideal plant because many of the proteins can be identified based on the homology to *Arabidopsis* or *Brassica* genes. On the other hand, it was evident that *Thlaspi* contains many proteins for which homology was not found from databases. These proteins may be of particular interest for further studies of metal tolerance, uptake, and accumulation.

15.11 HAIRY ROOTS OF HORSERADISH ARE AN IDEAL SYSTEM FOR INDUCTION OF PHYTOCHELATIN HOMOLOGS

When exposed to excess heavy metals, plants induce phytochelatins and related peptides (all designated as PCAs). Horseradish (*Armoracia rusticana*) was exposed for 3 days to cadmium (1

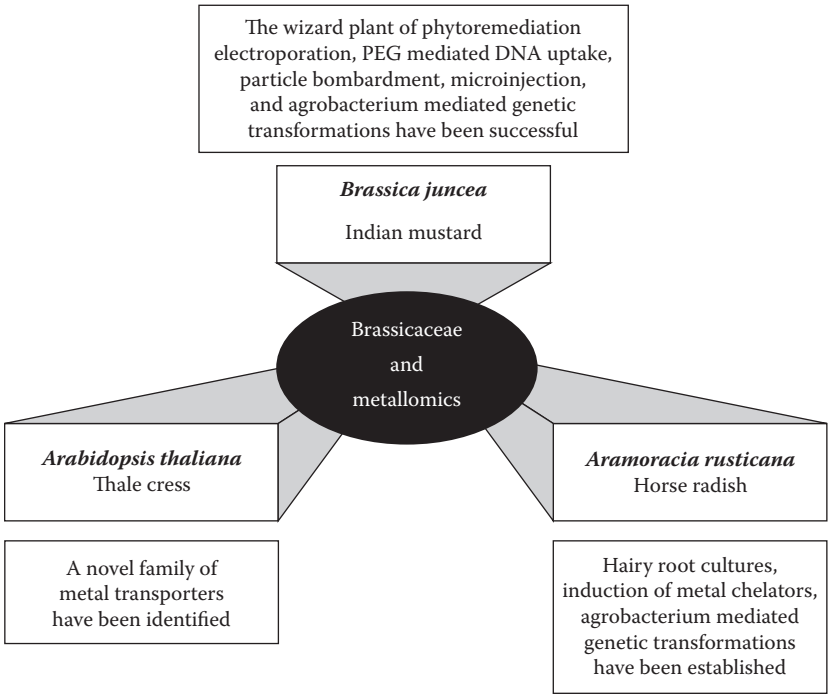

FIGURE 15.3 Biotechnology prospecting for phytoremediaiton of metals in the environment. In Brassicaceae *Arabidopsis thaliana*, *Brassica juncea*, and *Armoracia rusticana* have been extensively studied for metal sensitivity and resistance. In *A. thaliana*, a number of heavy metal accumulating and sequestering mutants have been identified. Brassicaceae are amenable to well-characterized biotechnological and molecular biological tools through which transgenic production can be achieved for field trials.

m*M*) along with reduced glutathione (2 m*M*); its hairy roots induced PCA. Brassicaceae are amenable for metallomics (Figure 15.3). The role of phytochelatins in sequestering toxic metals has been discussed in Chapter 16.

15.12 OVEREXPRESSION OF Γ-GLUTAMYLCYSTEINE SYNTHETASE IN INDIAN MUSTARD ENHANCED CADMIUM TOLERANCE AND ACCUMULATION

In an investigation of rate-limiting factors for glutathione and phytochelatin (PC) production and the importance of these compounds for heavy metal tolerance, Indian mustard (*Brassica juncea*) was genetically engineered to overexpress the *Escherichia coli* gene encoding g-glutamylcysteine synthetase (γ-ECS), targeted to the plastids. The γ-ECS transgenic seedlings showed increased tolerance to Cd and had higher concentrations of PCs, γ-GluCys, glutathione, and total nonprotein thiols compared with wild-type (WT) seedlings. When tested in a hydroponic system, γ-ECS mature plants accumulated more Cd than WT plants: shoot Cd concentrations were 40 to 90% higher. In spite of their higher tissue Cd concentration, the γ-ECS plants grew better in the presence of Cd than WT did. Thus, Zhu et al. [74] concluded that overexpression of γ-ECS increases biosynthesis of glutathione and PCs, which in turn enhances Cd tolerance and accumulation. Therefore, overexpression of g-ECS appears to be a promising strategy for the production of metal-tolerant plants for application in phytoremediation.

Nonprotein thiols (NPTs), which contain a high percentage of Cys sulfhydryl residues in plants, play a pivotal role in heavy metal detoxification. The reduced form of glutathione (γ-Glu-Cys-Gly,

GSH) is one of the most important components of NPT metabolism. GSH may play several roles in heavy metal tolerance and sequestration. It protects cells from oxidative stress damage, such as that caused by heavy metals in plants. PCs are heavy metal-binding peptides involved in heavy metal tolerance and sequestration [14]. PCs comprise a family of peptides with the general structure (γ-Glu-Cys)n-Gly, where n = 2 to 11 [30]. They were shown to be induced by heavy metals such as Cd in all plants tested [54], including Indian mustard [55]. The roles of GSH and PC synthesis in heavy metal tolerance were well illustrated in Cd-sensitive mutants of *Arabidopsis*. For example, the Cd-sensitive *cad2* mutant was defective in GSH and PC biosynthesis [21].

GSH is synthesized from its constituent amino acids in two sequential, ATP-dependent enzymatic reactions catalyzed by g-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GS), respectively. PC synthase subsequently catalyzes the elongation of the (γ-Glu-Cys)n by transferring a g-GluCys group to GSH or to PCs [65]. Genes encoding PC synthase have been cloned from plants and yeast [67–69]. The rate-limiting step for GSH synthesis in the absence of heavy metals is believed to be the reaction catalyzed by γ-ECS because the activity of this enzyme is feedback regulated by GSH and dependent on Cys availability [70]. This view was supported by the observation that overexpression of the *Escherichia coli GSHI* gene (which encodes γ-ECS) in poplar resulted in increased foliar GSH levels [71,72].

In contrast, overexpression of GS did not lead to an increase in GSH levels in poplar [73] or in Indian mustard [74] in the absence of heavy metals. However, the Indian mustard GS overexpressing plants showed higher levels of GSH and PC2 relative to untransformed plants in the presence of heavy metals. These GS plants also showed enhanced heavy metal tolerance and accumulation [74]. It has been reported that overexpression of tomato γ-ECS could restore some degree of heavy metal tolerance to the *cad2 Arabidopsis* mutant. However, overexpression of this gene did not increase the Cd tolerance of wild-type (WT) *Arabidopsis* plants. *E. coli* γ-ECS enzyme was overexpressed in the chloroplasts of Indian mustard. The transgenic γ-ECS plants were compared with WT Indian mustard plants with respect to their Cd accumulation and tolerance, as well as their levels of heavy metal-binding peptides.

15.13 OVEREXPRESSION OF MTS AS A MEANS TO INCREASE CADMIUM TOLERANCE

Plants overexpressing mammalian MTs were reported to be unaffected by concentrations of 100 to 200 μ*M* cadmium, but growth of *N. tabacum* control plants was severely inhibited at external cadmium concentrations of 10 μ*M* [77]. Transformants of *Brassica oleracea* expressing the yeast metallothionein gene CUP1 tolerated 400 μ*M* cadmium; however, wild-type plants were unable to grow at concentrations above 25 μ*M* cadmium in a hydroponic medium. Transformants grown at 50 μ*M* cadmium accumulated 10 to 70% higher concentrations of cadmium in their upper leaves than did nontransformed plants grown at 25 μ*M* cadmium. This indicates that the enhanced tolerance observed in the transgenic plants was unlikely to be a consequence of excluding cadmium from the leaves.

The concept of using plants to clean up contaminated environments is not new. About 300 years ago, plants were proposed for use in the treatment of wastewater. At the end of the 19th century, *Thlaspi caerulescens* and *Viola calaminaria* were the first plant species documented to accumulate high levels of metals in leaves. Members of the genus *Astragalus* were capable of accumulating selenium up to 0.6% in dry shoot biomass. Despite subsequent reports claiming hyperaccumulators, the existence of plants hyperaccumulating metals other than Cr, Ni, Mn, Se, and Zn has been questioned and requires additional confirmation [78]. The idea of using plants to extract metals from contaminated soil and the first field trial on Zn and Cd phytoextraction were conducted by Baker et al. [79].

In the 1990s, extensive research was conducted to investigate the biology of metal phytoextraction. Despite significant success, understanding of the plant mechanisms that allow metal extraction is still emerging. In addition, relevant applied aspects, such as the effect of agronomic practices on metal removal by plants, are largely unknown. It is conceivable that maturation of phytoextraction into a commercial technology will ultimately depend on the elucidation of plant mechanisms and application of adequate agronomic practices. Natural occurrence of plant species capable of accumulating extraordinarily high metal levels makes the investigation of this process particularly interesting.

15.14 OVEREXPRESSION OF GLUTATHIONE SYNTHETASE IN INDIAN MUSTARD ENHANCED CADMIUM TOLERANCE

An important pathway by which plants detoxify heavy metals is through sequestration with heavy metal-binding peptides called phytochelatins or their precursor, glutathione. To identify limiting factors for heavy metal accumulation and tolerance and to develop transgenic plants with an increased capacity to accumulate and/or tolerate heavy metals, the *Escherichia coli* gshII gene encoding glutathione synthetase (GS) was overexpressed in the cytosol of Indian mustard (*Brassica juncea*). The transgenic GS plants accumulated significantly more Cd than the wild type: shoot Cd concentrations were up to 25% higher and total Cd accumulation per shoot was up to threefold higher. Moreover, the GS plants showed enhanced tolerance to Cd at the seedling and mature plant stages. Cd accumulation and tolerance were correlated with the gshII expression level. Cd-treated GS plants had higher concentrations of glutathione, phytochelatin, thiol, S, and Ca than wild-type plants. The conclusion was that, in the presence of Cd, the GS enzyme is rate limiting for the biosynthesis of glutathione and phytochelatins and that overexpression of GS offers a promising strategy for the production of plants with superior heavy metal phytoremediation capacity.

Heavy metal pollution of soils and waters, mainly caused by mining and the burning of fossil fuels, is a major environmental problem. Heavy metals, unlike organic pollutants, cannot be chemically degraded or biodegraded by microorganisms. An alternative biological approach used to deal with this problem is phytoremediation — i.e., the use of plants to clean up polluted waters and soils [78,80]. Heavy metals or metalloids can be removed from polluted sites by phytoextraction, which is the accumulation of the pollutants in the plant biomass [81]. Compared with other remediation technologies, phytoremediation is less expensive (1000-fold less expensive than excavation and reburial of soil [82]) and is particularly suitable for treatment of large volumes of substrate with low concentrations of heavy metals. However, the presence of heavy metals inhibits plant growth, limiting the application of phytoremediation. Therefore, one trait of great significance to phytoremediation is the ability of plants to tolerate the toxic metals extracted from the soil [75].

15.15 CONCLUSIONS

In spite of several achievements in metallomics, certain limitations still prevail. Phytoremediation technology in using genetically engineered plants has several advantages. There have been apprehensions about dispersal of these contaminants if plants are edible through the food chain. In this regard, industrial crops, e.g., energy and fiber crops, would be the best alternative land use option. Industrial crop (*Helianthus annuus, Brassica juncea, Armoracia rusticana, Arabidopsis halleri, Gossypium hirsutum, Eucalyptus, Amaranthus, Cannabis sativa,* and *Linum usitatissimum*)-based phytoremediation systems would contribute to sustainable development. The feasibility of genetic manipulation via *in vitro* culture techniques needs to be exploited for the success of phytoremediation for sustainable development (Figure 15.4). However, future research must focus on various tools of metallomics for maintaining the high quality of the environment (Figure 15.5).

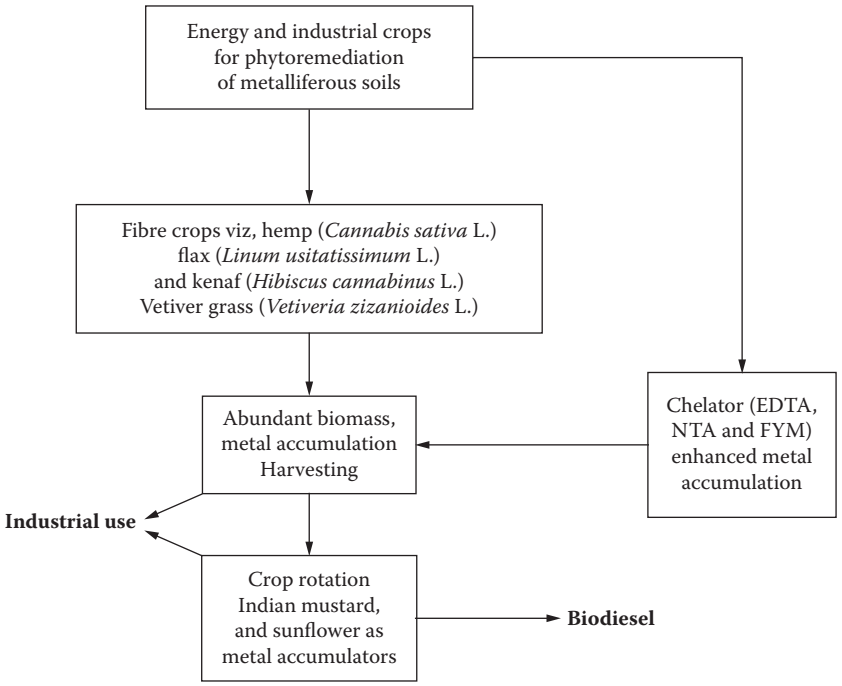

FIGURE 15.4 Industrial crops as potential tools of bioremediation for sustainable development. Sunflower for possible application in bioremediation of inorganic and organic pollutants.

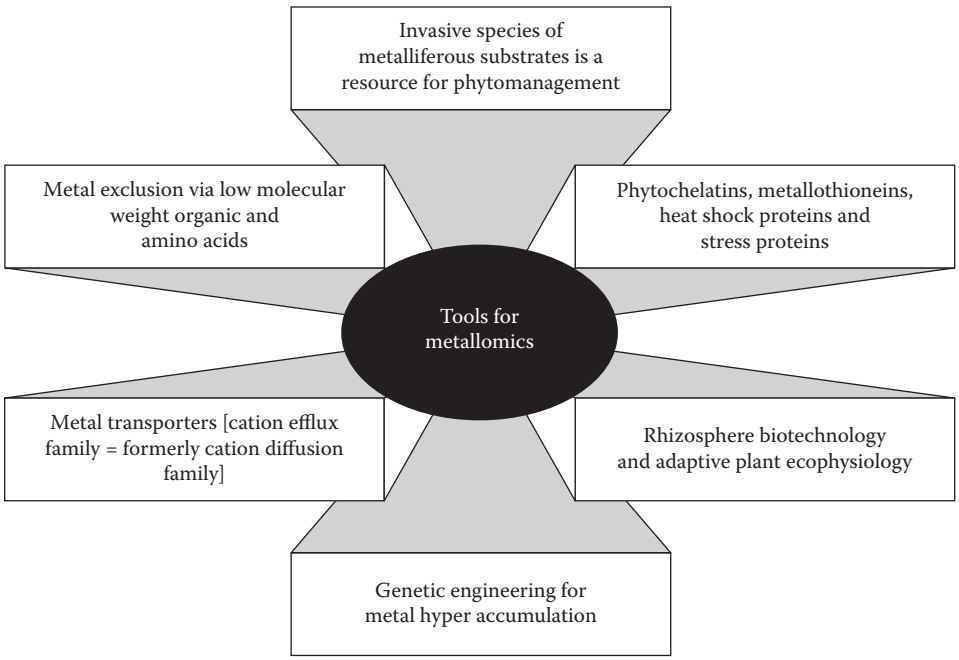

FIGURE 15.5 Potential tools for metallomics research.

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